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## EXPRESSION OF EPIDERMAL CAMP CHANGES IN PARALLEL WITH PERMEABILITY BARRIER STATUS

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### Abstract

Two critical defensive functions of the outer epidermis, the permeability barrier and antimicrobial defense, share certain structural and biochemical features. Moreover, 3 antimicrobial peptides (AMP); i.e., mouse beta-defensin 3 (mBD3), mouse cathelicidin protein (mCAMP), and the neuroendocrine peptide, catestatin, all localize to the outer epidermis, and both mBD3 and mCAMP are secreted from epidermal lamellar bodies with other organelle contents that subserve the permeability barrier. These 3 AMP are up-regulated in response to acute permeability barrier disruption, while conversely, mCAMP<sup>−/−</sup> mice (unable to combat gram-positive pathogens) also display abnormal barrier homeostasis. To determine further whether these two functions are co-regulated, we investigated changes in immunostaining for these 3 AMP in skin samples in which permeability barrier function in mice had been either compromised or enhanced. Compromised or enhanced barrier function correlated with reduced or enhanced immunohistochemical expression of mCAMP, respectively, but conversely with Cst expression likely due to the role of this AMP as an endogenous inhibitor of cathelicidin expression. mBD3 expression correlated with experimental barrier perturbations, but poorly with developmental changes in barrier function.

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These studies show that changes in cathelicidin and Cst expression parallel changes in permeability barrier status, with a less clear relationship with mBD3 expression.

## Keywords

Antimicrobial peptides; barrier repair; beta-defensin; calcipotriol; cathelicidin; catestatin; imiquimod; LXR; PPAR; permeability barrier; psychological stress; urea; vitamin D

## INTRODUCTION

The stratum corneum (SC) of mammalian epidermis mediates several critical protective functions (Elias, PM, 2005), two of which, maintenance of permeability barrier homeostasis and cutaneous antimicrobial defense (distal innate immunity), exhibit certain physical-chemical and biochemical features that contribute simultaneously to both functions (supplemental Table 1)(rev. in (Elias, PM, 2007, Elias, PM, *et al.*, 2008, Elias, PM, *et al.*, 2009)). For example, the low pH of the SC creates an ecological milieu that is both hostile to microbial pathogens, while simultaneously favoring growth of the normal flora (Aly, R, *et al.*, 1978, Korting, HC, *et al.*, 1990). Moreover, the highly cohesive and anhydrous characteristics of normal SC comprise a formidable physical barrier to invading microorganisms, while conversely pathogens invade between dyshesive corneocytes when the permeability barrier is compromised (Miller, SJ, *et al.*, 1988, Elias, PM, 2007). Furthermore, certain lipids that are required for the permeability barrier, such as free fatty acids (FFA) of both epidermal (Miller, SJ, *et al.*, 1988, Drake, DR, *et al.*, 2008) and sebaceous (Bibel, DJ, *et al.*, 1989, Georgel, P, *et al.*, 2005) origin, as well as the sphingoid bases of ceramides, also exhibit potent antimicrobial activity. Thus, the increased residence of *S. aureus* and other pathogens on lesions of atopic dermatitis (AD) could be explicable not only by alterations in barrier function and innate immunity (Radek, K, *et al.*, 2007), but also by the: i) high pH [cited in (Hatano, Y, *et al.*, 2009)]; ii) lipid-depleted extracellular matrix (Chamlin, SL, *et al.*, 2002); iii) reduced FFA/sphingosine content (Proksch, E, *et al.*, 2003)<sup>-</sup>(Arikawa, J, *et al.*, 2002); and iv) poor cohesion (Cork, MJ, *et al.*, 2006) of SC in lesional AD. Notably, the cathelicidin protein, hCAP18, and its carboxyterminal peptide, LL-37 also are down-regulated in lesional AD, explicable by increased Th2 signaling (Howell, MD, 2007) and/or excess serine protease activity (Morizane, S, *et al.*, 2010).

The link between permeability barrier status and antimicrobial defense is shown not only by their shared physical and biochemical characteristics, but also by the fact that acute perturbations in permeability barrier function stimulate metabolic responses that rapidly restore permeability barrier homeostasis in parallel with enhanced AMP expression; e.g., mCAMP, mBD3, catestatin (Cst), RNase 7, and psoriasin production all increase rapidly after acute barrier disruption (Elias, PM, *et al.*, 2005, Aberg, KM, *et al.*, 2008, Radek, KA, *et al.*, 2008, Glaser, R, *et al.*, 2009a). Conversely, mCAMP knock-out mice display abnormal permeability barrier homeostasis, demonstrating that cathelicidins are required for normal permeability barrier function (Aberg, KM, *et al.*, 2008). Notably, both the lipids that mediate permeability barrier function (Grayson, S, *et al.*, 1985), and at least 3 AMP; i.e., mCAMP (LL-37), mBD3(hBD2), and Cst expressed in the outer epidermis. Moreover, both

mCAMP (LL-37) and mBD3 (hBD2) are cargo within epidermal lamellar bodies (Oren, A, *et al.*, 2003, Braff, MH, *et al.*, 2005, Aberg, KM, *et al.*, 2007). Hence, their co-localization and presumed co-secretion insures that constituents of both the permeability and antimicrobial barriers are delivered in parallel to SC extracellular domains.

Our results suggest close, bidirectional changes in mCAMP expression under a variety of conditions where permeability barrier function is either compromised or enhanced, but an apparent, converse relationship with Cst expression, which could reflect its function as a  $\beta$ -muscarinic inhibitor of cathelicidin expression [(Radek, KA, *et al.*, 2010) and cited therein].

## RESULTS

### Permeability Barrier Status in Various Models

In normal mice, acute abrogations of the epidermal permeability barrier function, induced by either organic solvent applications or repeated tape strippings, provoke a transient decline in AMP levels, followed by rapid upregulation of expression of several AMP; i.e., mCAMP, mBD3, Cst, and psoriasin over 2–6 hrs in parallel with barrier restoration (Schroder, JM, *et al.*, 2006, Aberg, KM, *et al.*, 2008, Radek, KA, *et al.*, 2008, Glaser, R, *et al.*, 2009a). In these studies, we assessed changes in AMP expression in four situations in which permeability barrier homeostasis subnormal; i.e., after sustained *psychological stress* (PS) (Denda, M, *et al.*, 1998, Choi, EH, *et al.*, 2006); in young adult males (= *testosterone replete*) (Kao, JS, *et al.*, 2001); after *erythemogenic* UV-B exposure (Haratake, A, *et al.*, 1997), and in *chronologically (intrinsically) aged epidermis* (Choi, EH, *et al.*, 2007). Since our prior studies showed that PS downregulates both mCAMP and mBD3 expression (Aberg, KM, *et al.*, 2008), samples from PS mice served as positive controls for the other models. AMP status also was assessed in a library of paraffin-embedded materials from our previously-published studies where permeability barrier homeostasis had been altered either experimentally or different developmentally (Kao, JS, *et al.*, 2001) (Table 1) in testosterone-replete and chronologically-aged mice. In these studies, erythemogenic UV-B induced a dose- and time-dependent abnormality in permeability barrier function (see below), as reported previously (Haratake, A, *et al.*, 1997).

### Compromised Permeability Barrier Function Correlates Closely with Decreased mCAMP Expression

**Psychological Stress**—As reported previously, immunostaining for both mCAMP and mBD3 declined following PS [Fig. 1A; see also (Aberg, KM, *et al.*, 2007)]. Moreover, we now show further that Cst immunostaining also declines after short-term PS (i.e., 24–36 hrs), but Cst instead appears to normalize, or even supernormalize following exposure to more prolonged periods of PS (4 days of restraint) (Fig. 1B).

**Androgen Status (Gender)**—Previous studies have shown that testosterone-replete (adult) mice and humans display normal basal barrier function, but delayed permeability barrier recovery (Kao, JS, *et al.*, 2001) (Table 1). Therefore, we next compared epidermal mCAMP, mBD3, and Cst immunostaining in library skin samples from young adult male vs. female mice. While male mice display a marked decline in immunostaining for mCAMP,

they instead appear to display a modest enhancement of immunostaining for mBD3, and a marked increase in Cst expression (suppl. Fig. 1). These results suggest that the decline in permeability barrier with testosterone repletion is paralleled by a concomitant reduction in mCAMP, while mBD3 and Cst expression instead appear to increase in androgen-replete males.

**Erythemogenic UV-B**—While *suberythemogenic* doses of UV-B have been shown previously to enhance permeability barrier function (Hong, SP, *et al.*, 2008), *erythemogenic* UV-B instead provokes a transient, delayed (by 48–96 hrs), and dose-dependent, barrier abnormality, as we reported previously (Haratake, A, *et al.*, 1997) (Fig. 2). Therefore, we next examined whether erythemogenic UV-B produces parallel alterations in AMP expression in mice. In these studies, the intensity of AMP immunostaining was quantitated by a blinded observer on multiple, pooled, coded images at each time point. Erythemogenic UV-B (5 MED) provokes a progressive decline in mCAMP levels which returns towards normal at day 5 (Fig. 2; suppl. Fig. 2A). In contrast, erythemogenic UV-B did not alter mBD3 immunostaining (suppl. Fig. 2B), while it simultaneously stimulated a sustained increase in Cst expression immediately after exposure, with immunostaining remaining elevated until day 5, when immunostaining began to decline (Fig. 2; suppl. Fig. 2C). Together, these results suggest that the transient defect in permeability barrier function, provoked by erythemogenic UV-B, is paralleled by a marked decline in mCAMP, a minimal decline in mBD3, but a marked enhancement of Cst expression.

**Chronologically-Aged Mouse Skin**—Permeability barrier homeostasis progressively declines during chronologic aging (Ghadially, R, *et al.*, 1995, Choi, EH, *et al.*, 2007) (Table 1). Therefore, we next examined age-related abnormalities in AMP expression in library tissue samples from young vs. moderately-aged mouse epidermis (15–18 mos), analogous to human age 50–65 years (Choi, EH, *et al.*, 2007). Under basal conditions, epidermis of young mice displays low constituent levels of immunostaining for both mCAMP and mBD3, with a prominent decline in mCAMP immunostaining in chronologically aged mouse epidermis. In contrast, both mBD3 and Cst levels instead appears to markedly increase in aged mouse epidermis (suppl. Fig. 3).

### Improved Permeability Barrier Function Correlates with Enhanced mCAMP Expression

**Imiquimod and Calcipotriol Treatment**—Both the immune-enhancer, imiquimod (IMQ) and the 1,25(OH)<sub>2</sub> vitamin D3 analogue, calcipotriol, improve barrier function under a variety of experimental and clinical conditions (Barland, CO, *et al.*, 2004). Therefore, we next delineated the effects of repeated applications of topical IMQ or calcipotriol on mCAMP expression in normal mouse epidermis. Untreated murine epidermis again clearly demonstrated low, but readily-detectible immunostaining for both mCAMP and mBD3, localized to the outer epidermis (Aberg, K, *et al.*, 2007, Aberg, KM, *et al.*, 2008). Although both IMQ and calcipotriol treatments appeared to increase immunostaining for mBD-3 and mCAMP in comparison to vehicle alone, the increase in mCAMP appeared to be greater than that achieved in parallel, calcipotriol-treated mice (suppl. Fig. 4A vs. 4B). The increase in mCAMP and mBD3 in calcipotriol- and in IMQ-treated mice displays a linear pattern in the SC, corresponding to membrane domains, and it also further localized to vesicles in the

cytosol of stratum granulosum (SG) cells (suppl. Fig. 4B, insert, arrows), consistent with its known localization in epidermal lamellar bodies (Oren, A, *et al.*, 2003, Aberg, K, *et al.*, 2007). Finally, we examined AMP expression after several other unrelated maneuvers, previously shown to enhance barrier function. In each of these examples, mCAMP expression inevitably increased, but mBD3 and Cst did not always change in parallel (Table 2). Together, these results demonstrate first that IMQ and calcipotriol treatment appear to increase expression of both mCAMP and mBD3 in the outer epidermis. Second, several other, unrelated approaches that improve barrier function also enhance mCAMP expression, with more variable results for mBD3 and Cst (not shown).

## DISCUSSION

We addressed here the hypothesis that permeability barrier function and antimicrobial defense are integrated and co-regulated functions (Elias, PM, 2005), examining whether either experimental perturbations or developmental changes that either reduce or enhance permeability barrier status are accompanied by parallel changes in epidermal AMP expression. The impetus for these studies came first, from prior work that showed that these two functions are co-regulated and interdependent in normal epidermis (Aberg, KM, *et al.*, 2008, Hong, SP, *et al.*, 2008, Proksch, E, *et al.*, 2008); and second, that at least one perturbant of the permeability barrier (psychological stress) down-regulates mBD3 and mCAMP expression (Aberg, KM, *et al.*, 2007). Several studies already have shown that the converse is true; e.g., epidermal AMP expression, including Cst expression (Radek, KA, *et al.*, 2008), increase after acute barrier insults in parallel with barrier recovery (Elias, PM, *et al.*, 2005, Aberg, KM, *et al.*, 2008), and after blockade of both glucocorticoid (GC) production and action in PS mice (Aberg, KM, *et al.*, 2007). We extend these prior observations here by showing first, that short-term PS reduces not only mCAMP (LL-37) and mBD3 (hBD2) expression, but as PS is prolonged, Cst expression also begins to decline.

Our results provide several additional examples to support a putative relationship between permeability barrier and antimicrobial status, at least for mCAMP. *Testosterone depletion* by either surgical or medical means improves permeability barrier function, while conversely, testosterone-replete mice and humans display diminished permeability barrier function (Kao, JS, *et al.*, 2001). While we showed here an apparent, parallel decline in mCAMP expression in males vs. females, immunostaining for both mBD3 and Cst instead appeared to increase in the epidermis of adult male mice. Thus, it is possible that changes in androgen status could impose potentially-important variations in cutaneous antimicrobial defense.

*Suberythemogenic doses* of UV-B have been shown to upregulate permeability barrier function and mBD3/mCAMP expression simultaneously (Aberg, KM, *et al.*, 2007), further supporting a putative relationship between these two functions. Conversely, we now show here that *erythemogenic* doses of UV-B that compromise permeability barrier function (Haratake, A, *et al.*, 1997) also markedly appear to down-regulate mCAMP, but produce only minimal, transient alteration in mBD3 expression. The progressive decline (and recovery) of mCAMP expression parallels the time course over which the permeability barrier defect evolves and then recovers. Yet, Cst expression instead appeared to increase at all time points after erythemogenic UV-B. Our prior studies showed that the UV-B-induced

permeability barrier abnormality correlates with passage of a band of secretion-incompetent, apoptotic cells through the stratum granulosum-SC interface (Holleran, WM, *et al.*, 1997). While such a toxic mechanism could contribute to the observed decline in production of mCAMP, it certainly did not impede Cst expression. Thus, toxicity alone likely cannot account for the selective decline in mCAMP expression after UV-B expression. The anti-parallel changes in Cst can be explained instead by its role as a muscarinic inhibitor of cathelicidin expression (Radek, KA, *et al.*, 2008). Thus, these studies further support a close link between UV-B-induced changes in permeability barrier function and cathelicidin expression. Furthermore, together with the work of Hong, et al. on suberythemogenic UV-B (Hong, SP, *et al.*, 2008), these studies have potential clinical implications about how UV-B irradiation should be deployed for the treatment of inflammatory dermatoses. While current recommendations propose 'pushing' UV-B phototherapy doses upwards into the erythemogenic range, this approach clearly could pose adverse consequences not only for both permeability barrier function, but also for cutaneous antimicrobial defense.

Permeability barrier function begins to decline in adult humans above the age of 50 (Choi, EH, *et al.*, 2007), becoming further compromised above age 75 (Ghadially, R, *et al.*, 1995), and/or with superimposed photoaging (Reed, JT, *et al.*, 1997). We showed here that 15–18 mos old mice, analogous to humans over age 50, displayed reduced mCAMP levels, while both mBD3 and Cst immunostaining instead appeared to increase in this age group (Table 2). In attempting to explain these, and other divergent results for mCAMP vs. mBD3, it should be noted that these families of AMP are regulated by entirely different mechanisms (Oren, A, *et al.*, 2003, Braff, MH, *et al.*, 2005, Choi, EH, *et al.*, 2005, Aberg, KM, *et al.*, 2007, Peric, M, *et al.*, 2008, Eyerich, K, *et al.*, 2009). While endogenous 1,25(OH)<sub>2</sub> vitamin D3 and other VDR ligands regulate cathelicidin expression (Zasloff, M, 2005, Elias, PM, 2007, Aberg, KM, *et al.*, 2008, Drake, DR, *et al.*, 2008, Hong, SP, *et al.*, 2008, Schaubert, J, *et al.*, 2008), a variety of cytokines instead stimulate  $\beta$ -defensin production (Nomura, I, *et al.*, 2003, de Jongh, GJ, *et al.*, 2005, Elias, PM, *et al.*, 2005, Yano, S, *et al.*, 2008, Kobayashi, M, *et al.*, 2009). Accordingly, endogenous vitamin D levels typically decline with age (Holick, MF, 1987), perhaps accounting for decrease in mCAMP levels are observed here in aged murine epidermis. In contrast, cytokine levels vary widely during aging (Ye, J, *et al.*, 1999, Corsini, E, *et al.*, 2009), but IL-1 $\alpha$  levels in particular decline with chronologic aging, and are associated with decreased epidermal lipid production (Ye, J, *et al.*, 1999, Barland, CO, Elias, P.M., Ghadially, R., 2005). In contrast, other epidermal cytokines (e.g., TNF $\alpha$ ) instead increase in aged epidermis (Corsini, E, *et al.*, 2009), consistent with our observation that mBD3 immunostaining persists, or even increases in moderately-aged mouse skin. The basis for the apparent, age-related increase in Cst expression is unclear at present, but it could again relate to the role of this neuropeptide as an endogenous inhibitor of cathelicidin production. Whether further abnormalities in antimicrobial defense occur in moderately-aged epidermis, and/or with still more-advanced aging and/or photoaging is not yet known. Nevertheless, these age-related differences in AMP expression, which do not strictly parallel changes in permeability barrier status, could also have important clinical implications, since they suggest that cutaneous antimicrobial defense becomes compromised relatively early during the aging process.



We also examined here the opposite situation, asking whether maneuvers that are known to enhance barrier function also upregulate AMP expression. The immune response modifier, imiquimod (IMQ), acts through two members of the toll-like receptor (TLR) family, TLR7 and/or 8, which recognize microbial pathogens or their metabolic products, and function as primary sensors of the innate immune system (Ambach, A, *et al.*, 2004, Sauder, DN, 2004, Lai, Y, *et al.*, 2008). These TLR are cell surface receptors that, when activated, stimulate production of epidermis-derived, interferon-alpha, tumour necrosis factor, and interleukin-1 $\alpha$  (Sauder, DN, 1990, Barland, CO, *et al.*, 2004, McInturff, JE, *et al.*, 2005, Takeuchi, O, *et al.*, 2009). We have shown topical IMQ enhances barrier function in normal and aged epidermis, through stimulating IL-1 $\alpha$  production, which in turn, stimulates epidermal lipid synthesis (Ye, J, *et al.*, 1999, Barland, CO, *et al.*, 2004). Since human  $\beta$ -defensins are upregulated by multiple cytokines, it is highly likely that hBD2 (mBD3) upregulation by topical IMQ is signalled by epidermal production of cytokines. Yet, while the apparent increase in mBD3 immunostaining after calcipotriol treatment was unexpected, it could be linked to well-known effects of VDR ligands on epidermal differentiation (Bikle, DD, *et al.*, 2010). Finally, we examined changes in AMP expression in two other unrelated situations where barrier function is enhanced; i.e., after treatment with topical 5–20% urea (Grether-Beck, S, *et al.*, 2011), and after topical applications of Chinese herbal medications to normal mouse skin (Man, MQ, *et al.*, 2008, Man, M, *et al.*, 2011). In both of these situations, mCAMP expression increased in parallel with enhanced permeability barrier function (Fig. 3 and Table 2).

## MATERIALS AND METHODS

### Models with Compromised Permeability Function

**1) Psychological stress**—Our prior studies have shown that both sustained psychological stress (PS) and exogenous glucocorticoids (GC) downregulate barrier function (Denda, M, *et al.*, 2000, Choi, EH, *et al.*, 2005) in parallel with reduced expression of both mCAMP and mBD3 (Aberg, KM, *et al.*, 2007). Hence, library samples of biopsies from PS (short-term) served as positive controls for the additional conditions studied here, where barrier function also is compromised.

Male hairless mice (Skh1/hr) were purchased from Charles River Laboratories (Wilmington, MA). To assess the effects of more long-term PS, animals were placed in motion-restricted environments for 12 hrs once daily during night time for 72–96 hrs. Food and water were restricted in parallel in a control non-motion-restricted group. A plastic container (4.0 [W]  $\times$  3.0[H]  $\times$  11.5 [L] cm<sup>3</sup>) with mesh walls on the top were used for PS environments, of which the inner space was minimized to allow animals to rotate their bodies. All animals were studied between 8 and 10 weeks of age. The animal experiments described in this study were conducted in accordance with accepted standards of humane animal care, under protocols approved by the local institutional animal care and use committee at the San Francisco VA Medical Center.

**2) Testosterone-Replete (adult male vs. adult female) Mice**—To assess the impact of physiologic levels of testosterone, previously shown to compromise permeability barrier



function (Kao, JS, *et al.*, 2001), we compared AMP expression in library samples of adult male vs. female mice (aged 8–10 wks; n=4 each), processed for immunofluorescence, as described below. Serum testosterone levels were > 500 pg per ml in male animals, and < 200 pg per ml in the female animals (Kao, JS, *et al.*, 2001).

**3) Erythemogenic UVB Exposure**—Hairless, 8–10 week old female hairless mice were purchased from Charles River Laboratories (Wilmington, MA), and fed Purina mouse diet (Ralston Purina Co, St. Louis, MO, USA) and water *ad libitum*. Natural sunlight was excluded and animals were exposed only to low levels of incandescent light prior to UV-B irradiation. UV-B irradiation was delivered with Phillips TL20W/12 fluorescent lamps (Eindhoven, Netherlands), emitting 280–320 nm. The dorsal skin of each mouse was either sham-irradiated, or irradiated with single dose equivalents of either five (5) or ten (10) minimal erythral doses (MED) (n=5 each). One MED, previously determined on the same strain of mice, equals approximately 20 mJ/cm<sup>2</sup> (60–100 mJ/cm<sup>2</sup>/hr equals 1 MED in humans with type II/III pigmentation). Twenty animals were treated in each group, and samples were taken before, immediately after, and then 1, 3, and 5 days following UV-B exposure, followed by processing for immunofluorescence studies (see below).

**4) Chronologically Aged Mouse Skin**—Epidermal AMP expression was compared in library samples from aged (15–18 mos, equivalent to an age range of 50–60 in humans) vs. young adult (3–4 mos) hairless mice (Skh1, Jackson Labs; n=4 each) (Choi, EH, *et al.*, 2007). The analogous age of mice and humans was determined from optimal life spans (≈120 years in humans and 24 months in mice). Hairless mice begin to display a progressive permeability barrier abnormality after 15 months (Ghadially, R, *et al.*, 1995, Choi, EH, *et al.*, 2007).

### Models with Enhanced Permeability Barrier Function

Not only blockade of GC production/action (Aberg, KM, *et al.*, 2007), but also suberythemogenic UV-B have already been shown to stimulate mCAMP and mBD3 production (Hong, SP, *et al.*, 2008, Glaser, R, *et al.*, 2009b) (Table 1). Here, we assessed changes in mCAMP expression after several additional approaches that enhance barrier function. We focused on changes in mCAMP in this subset of studies, because it most closely paralleled changes in barrier status in the previously-assessed models with reduced function.

**1) Imiquimod and Calcipotriol**—Prior studies have shown that both 1,25 (OH)<sub>2</sub> vitamin D3 and its analogues (Bikle, DD, 2010), as well as imiquimod (Barland, CO, *et al.*, 2004), enhance barrier function in a variety of settings. The dorsal skin of each mouse was treated with either topical IMQ (Aldara®) 5% cream, calcipotriol (Dovonex®) cream 50 µg/g, or vehicle twice daily for 7 days (n=4 mice in each group). Parallel control groups of hairless mice were treated with the vehicles for the equivalent drug alone at the same time points.

Library biopsy samples from comparable cohorts of 4–5 normal hairless mice each also were assessed after the following approaches that are known to enhance barrier function:

**2) Chinese herbal mixture and urea**—We recently showed that various Chinese herbal mixture improve barrier function in normal hairless mice (Man, MQ, *et al.*, 2008, Man, M, *et al.*, 2011). Recent studies also have shown that topical urea at concentrations 5% improves barrier function in normal human and mouse skin (Grether-Beck, S, *et al.*, 2011).

### Tissue Processing and Immunofluorescence

Biopsies for immunostaining were obtained at time points when maximal changes in barrier function occurred (see figure and table legends, as well as cited references for further details). Full-thickness skin biopsies, that had been either snap-frozen in liquid nitrogen or library samples embedded in paraffin, were utilized for immunofluorescence studies. Frozen sections (5 µm) were soaked in acetone for 10 min, washed in PBS, and blocked with 4% BSA and 0.5% cold-water fish gelatin in PBS for 30 min. 10 µm paraffin-embedded tissue sections were de-paraffinized, rehydrated and then rinsed with de-ionized water, followed by three washes in PBS. Sections were incubated for 30 min in blocking buffer (4% bovine serum albumin, 0.5% cold water fish gelatin in PBS), and then incubated overnight at 4°C with the primary antibodies in blocking buffer. The next morning, sections were washed 3 times in PBS and incubated for 40 min at room temperature with the Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody, diluted 1:2,000 in blocking buffer. Slides then were incubated overnight at 4°C with primary antibodies (1:500 or 1:1,000) against either catestatin (from Phoenix Labs and Richard Gallo, UCSD), mBD-3 (Alpha Diagnostics), or mCAMP (from Dr. Richard Gallo, UCSD), followed by incubation with FITC-conjugated, goat anti-rabbit secondary antibody (Alpha Diagnostics) for 45 min at room temperature, as described (Aberg, KM, *et al.*, 2007, Radek, KA, *et al.*, 2008). Sections were counterstained with propidium iodide and visualized on a Leica TCS-SP Laser confocal microscope at excitation and emission wavelengths of 488 and 532 nm, respectively, photographed at 40× and the intensity of AMP immunostaining was scored blindly in randomly-mixed micrographs (n=20 in each group) as either 0 (subnormal), 1 (normal=basal), 2–5 increased, with 5 (= most intense, antigen-positive immunostaining). Sections labeled with only the secondary antibody, and/or sections from mCAMP ko mice (Nizet, V, *et al.*, 2001, Aberg, KM, *et al.*, 2008) served as controls.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Abbreviations

<b>AMP</b>	Antimicrobial peptide
<b>AD</b>	atopic dermatitis

<b>BD</b>	$\beta$ -defensin
<b>Cst</b>	castestatin
<b>FFA</b>	free fatty acids
<b>GC</b>	glucocorticoid
<b>IMQ</b>	imiquimod
<b>mCAMP</b>	mouse cathelicidin antimicrobial peptide
<b>MED</b>	minimal erythema dose
<b>PS</b>	psychological stress
<b>SC</b>	stratum corneum

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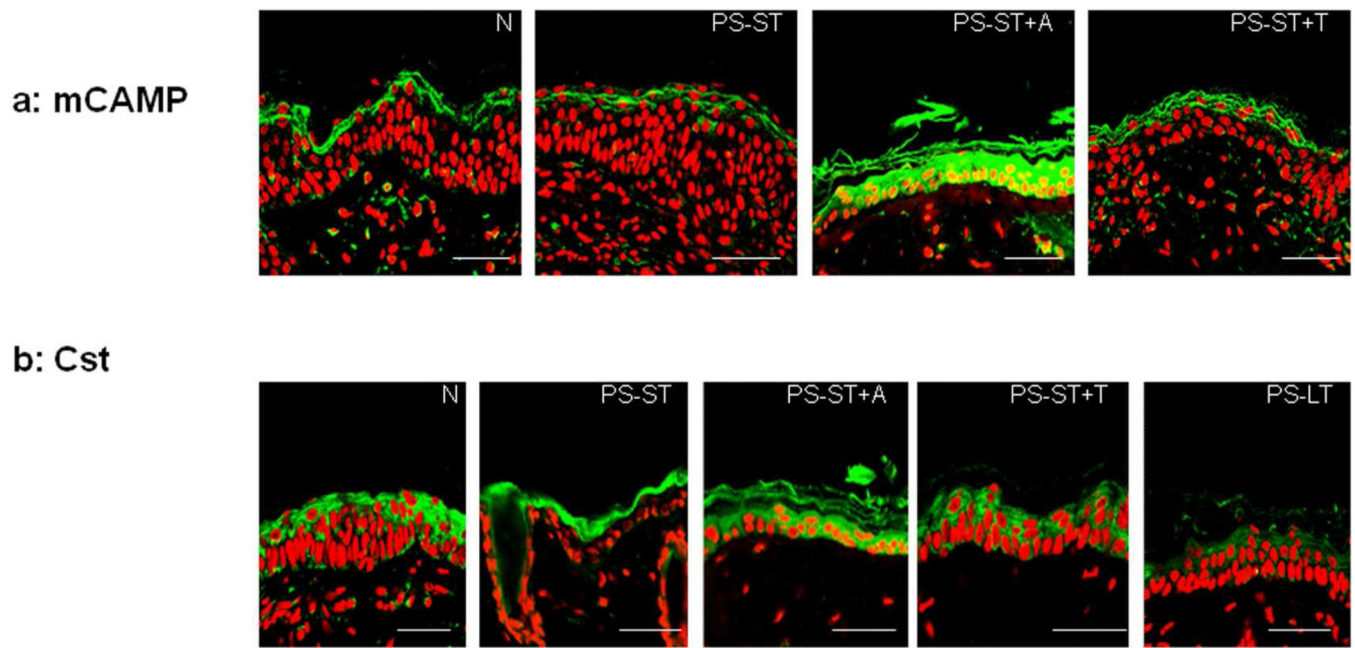
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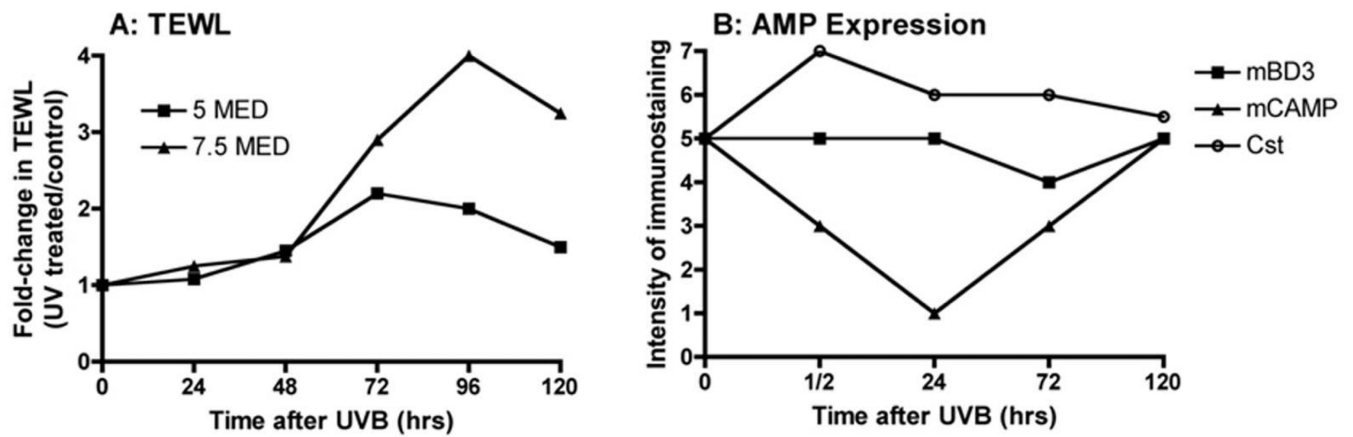
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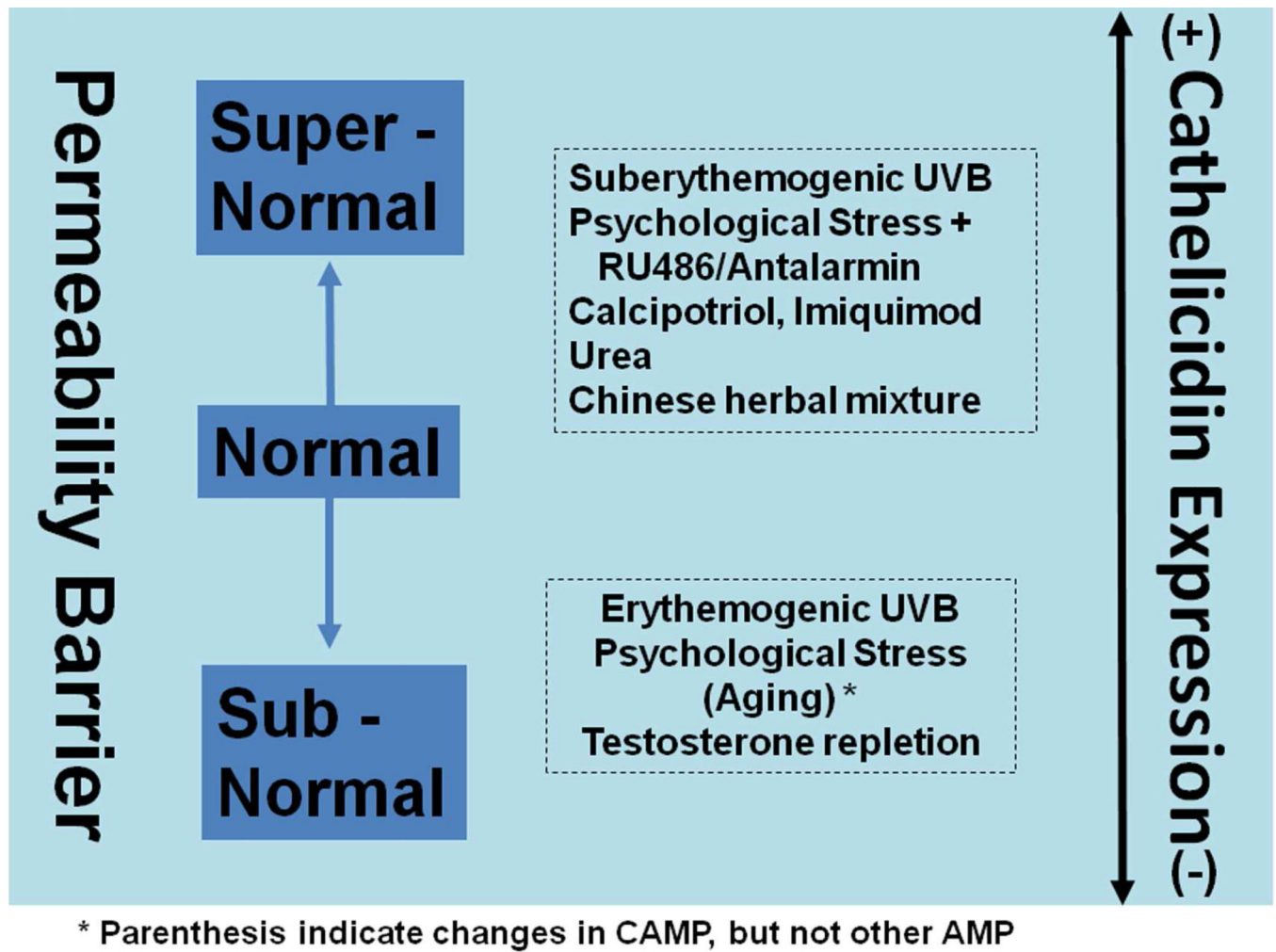
**Figure 1. Psychological Stress (PS) Decreases Immunostaining for mCAMP, mBD3 and Cst in Both a GC and a  $\beta$ -Adrenergic-Dependent Manner**

Hairless mice (n=4 or 5 each) were exposed to either insomnia-induced PS for 36–48 hrs (short-term PS, PS-ST) or restraint-induced stress for 96 hrs (long-term PS, PS-LT), while parallel groups of PS mice (n=4 or 5 each) were co-treated with intraperitoneal antalarmin or Ru486 [not shown; see (Aberg, KM, *et al.*, 2008)], or topical timolol (T) (0.38% in saline) (see Methods for further details). Five  $\mu$ M frozen sections were labeled with primary antibodies against mCAMP, mBD3, or Cst. Propidium iodide was used to counterstain nuclei. Green immunostaining represents AMP labeling; Mag bars in these and all subsequent figures = 40  $\mu$ m.



**Figure 2. Quantitation of Decline in mCAMP Immunostaining Parallels Development of a Permeability Barrier Abnormality**

A: UV-B-induced changes in permeability barrier function are modified from (Haratake, A, *et al.*, 1997). B: Micrographs ( 10 each) from mice treated with erythemogenic UV-B (n=4, as in suppl. Fig. 2) were coded, randomized and graded according to the intensity of staining for mCAMP, mBD3, and Cst by a blinded observer.



**Figure 3. Summary of Results – Maneuvers That Alter Barrier Functions Are Paralleled by Bidirectional Changes in Cathelicidin Expression**

**Table 1**

Changes in barrier function in various mouse models

Barrier Perturbant	Basal Barrier Function	Barrier Recovery Kinetics
Psychological stress	Declines <sup>a,b</sup>	Delayed <sup>a,b</sup>
Testosterone-replete (male)	Declines <sup>c</sup>	Delayed <sup>c</sup>
Erythemogenic UV-B (5–10 MED)	See Figure 5B <sup>d</sup>	Delayed <sup>d</sup>
Intrinsic Aging	Declines <sup>e,f</sup>	Delayed <sup>f</sup>
<b>Improved Barrier</b>		
Sub-erythemogenic UV-B	Improves <sup>g</sup>	Accelerates <sup>g</sup>
Calcipotriol	Improves <sup>h</sup>	N/D
Endogenous GC Blockade	Improves <sup>i</sup>	Accelerates <sup>a,b</sup>
Imiquimod	Improves <sup>j</sup>	Accelerates <sup>j</sup>
Triple lipids	Improves <sup>k</sup>	Accelerates <sup>k</sup>
Petrolatum	N/D	Accelerates <sup>k</sup>
PPAR $\alpha$	No changes	Accelerates <sup>l</sup>
LXR	No changes	Partially normalizes <sup>m</sup>
Chinese herbal mixture	No changes	Accelerates <sup>n</sup>
Urea	Improves	N/D

<sup>a</sup>Denda, et al., Am J Phys Reg Integ Comp Phys 278: R367–72, 2000;<sup>b</sup>Choi, et al., Am J Phys Reg Integ Comp Phys 291:R1657–62, 2006;

Kao, et al., J Inv Derm 116:443–451, 2001;

<sup>d</sup>Haratake, et al., J Inv Derm 108:769–775, 1997;<sup>e</sup>Ghadially, et al., J Clin Inv 95: 2281–90, 1995;<sup>f</sup>Choi, et al., J Invest Derm 127:2847–2856, 2007;

Hong, et al., J Inv Derm 128: 2880–7, 2008;

<sup>h</sup>Bikle, J Bone Min Metab 28: 117–30, 2010;<sup>i</sup>Aberg, et al., J Clin Inv 117: 3339–49, 2007;<sup>j</sup>Barland, et al., J Inv Derm 122: 330–6, 2004;<sup>k</sup>Man, et al., Arch Derm 131: 809–16, 1995;<sup>l</sup>Mao-Qiang, et al., J In Derm. 123:305–12, 2004;<sup>m</sup>Kömüves, et al., J Inv Derm. 118:25–34, 2002;<sup>n</sup>Man, et al., Exp Derm. In Press, 2010;<sup>o</sup>Grether-Beck, et al.; In Press, 2011.

**Table 2**

Permeability Barrier Status	AMP Expression		
Decreased	mCAMP	mBD3	Cst
Psychological Stress (PS)	↓	↓	↑
Exogenous GC	↓	↓	N/D
Testosterone-replete	↓	No change	No change
Erythemogenic UV-B	↓	(↓)	↑↑
Aging	↓	↑	↑
Increased			
PS + Ru486/Antalarmin	↑ <i>a</i>	↑	↑
Sub-erythemogenic UV-B	↑ <i>b</i>	↑ <i>b</i>	N/D
Imiquimod	↑	↑	N/D
Chinese Herbal Medicine	↑	↑	N/D
Calcipotriol	↑	↑	N/D
Urea	↑	↑	N/D

<sup>a</sup> Aberg, et al., J Clin Inv 117:3339–49, 2007,

<sup>b</sup> Hong, et al., J Inv Derm 128:2880–7, 2008.

N/D = Not demonstrated